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WITNESS my hand this
Twenty-third day of June 2000

K. Ward

KAY WARD
TEAM LEADER EXAMINATION
SUPPORT AND SALES

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AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Applicant(s):

THE UNIVERSITY OF MELBOURNE

Invention Title:

NEUROTROPHIN AGONISTS

The invention is described in the following statement:

involved in several types of sensory neuropathy (Lindsay, 1994).

The biological effects of BDNF and the other neurotrophins are mediated by binding to two classes of cellular receptor: members of the trk family of receptor tyrosine kinases, and the low affinity neurotrophin receptor, p75. Specific neurotrophins bind with high affinity (K_d approximately 10^{-11} M) to particular trk members expressed by responsive neurons: thus NGF and NT-3 bind to trkA; BDNF and NT-4/5 bind to trkB; NT-3 binds to trkC. Binding of a neurotrophin to its specific trk receptor causes receptor homodimerisation, triggering the intrinsic kinase domains of the receptors to autophosphorylate intracellular tyrosine residues, and thus initiating signal transduction cascades leading to neuronal survival (Barbacid, 1994). In contrast, p75 acts as a common low affinity receptor for the neurotrophins, and binds each with comparable affinity (K_d approximately 10^{-9} M); p75 is expressed widely on central and peripheral neurons as well as on other cell types, such as Schwann cells (for review see Chao and Hempstead, 1995).

While the role of the trk members in signalling the neurotrophic effects of the neurotrophins is well established, the function of p75 remains controversial. Although there is compelling evidence that p75 either modulates responses mediated by trk members or itself plays a part in survival signalling, the final effect of p75 appears to depend on the relative levels of expression of p75 and trk (Kaplan and Miller, 1997). Of particular interest are the observations that p75 may, under certain circumstances, cause apoptosis either in the absence (Rabizadeh *et al*, 1993; Barrett and Bartlett, 1994) or presence (Frade *et al*, 1996) of bound neurotrophin. This "death signal" of p75 may be mediated by an intracellular region homologous to the death-signalling domains of tumour necrosis factor (TNF) receptor-1 and Fas (Chapman, 1995).

accessible, and together form a binding surface that almost exclusively spans the top half of the molecule.

Other site-directed mutagenesis studies have shown that three positively-charged residues in each of the neurotrophins are of paramount importance in binding to p75 (Ibáñez *et al*, 1992; Rydén *et al*, 1995). These data are consistent with the idea that p75 shares a common binding interface with the neurotrophin family. There are, however, differences in the position of these three residues in different neurotrophins: in NGF, NT-3 and NT-4/5, the three positively-charged residues are spread across two adjacent loops, while in BDNF the three positively-charged residues are contiguous amino acids (Lys^{95}-Lys^{96}-Arg^{97}), located on loop 4 (Figure 1).

The ability of exogenously administered neurotrophic factors such as BDNF to rescue neurons in a variety of *in vivo* models of neurodegeneration has led to the widespread belief that neurotrophins and other neurotrophic factors offer exciting prospects for the treatment of neurodegenerative diseases, such as motor neuron disease and peripheral neuropathies (for review see Hefti, 1994). Unfortunately, because they are proteins, neurotrophic factors are orally inactive, are unable to cross the blood-brain barrier, and typically have a short half-life in plasma (Dittrich *et al*, 1994). Thus the recombinant human neurotrophic factors themselves are unlikely to be optimal agents for the long-term treatment of neurodegenerative disease. Indeed, the lack of success thus far of neurotrophic factors in clinical trials for the treatment of motor neuron disease has been attributed to the inability of the proteins to reach their targets in the central nervous system (CNS) following subcutaneous administration (Penn *et al*, 1997). One means of circumventing these problems would be to develop low molecular weight, non-peptidic analogues of neurotrophic factors with improved pharmacokinetic characteristics.

in some of the dimers found to be active is incorporated at sites other than those predicted to be optimal on the basis of the structure-activity relationships. In addition, the linker distance found to give optimal activity was shorter than that predicted from our design template.

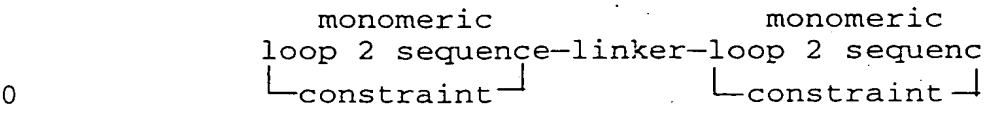
By combining these data, we have been able to create a novel class of tricyclic dimeric peptides with markedly improved potency over the dimeric peptides.

In contrast, monomeric cyclic peptides based on the p75-binding tripeptide sequence found in loop 4 had no inhibitory effects on the neuronal survival activity of either BDNF or NGF (Zwar and Hughes, 1997). To our surprise, however, when tested in the absence of neurotrophin one of these monomeric peptides acted as a BDNF-like agonist, able to promote the survival of chick sensory neurons in culture.

Summary of the Invention

According to a first aspect, the invention provides a cyclic compound of one or more cyclic moieties, which has a biological activity of brain-derived neurotrophic factor (BDNF).

In one embodiment, the compound is a bicyclic dimeric compound (that is, a compound composed of two monocyclic compounds connected by a chemical linker) based on loop 2 of BDNF, of general formula:

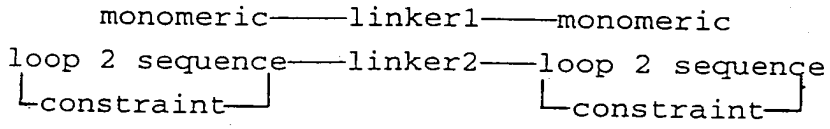


where:

"monomeric loop 2 sequence" represents a sequence of amino acid residues or functional equivalents thereof, which is sustantially homologous to the loop 2 region of BDNF, and comprises all or part of the following sequence:

(iii) cyclising via the formation of an amide bond between a side chain (for example of a lysine or aspartate residue) and either the C-terminal carboxyl or N-terminal amine, either directly or a spacer group as described in (i) above. The residues contributing the side chains may be derived from the monomeric loop 2 sequence itself, or may be incorporated into or added on to the monomeric loop 2 sequence for this purpose.

In a second embodiment, the compound is a tricyclic dimeric compound (that is, a compound composed of two monocyclic compounds connected by two chemical linkers) based on loop 2 of BDNF of general formula:



where:

"monomeric loop 2 sequence" is a sequence of amino acid residues or functional equivalents thereof, which is substantially homologous to the loop 2 region of BDNF, and comprises all or part of the following sequence: Glu^{40}-Lys^{41}-Val^{42}-Pro^{43}-Val^{44}-Ser^{45}-Lys^{46}-Gly^{47}-Gln^{48}-Leu^{49}-Lys^{50}-Gln^{51};

"constraint" represents a conformational constraint imposed on the monomeric loop 2 sequence, which acts to limit the flexibility of the monomeric loop 2 sequence, for example by covalently linking all or part of the monomeric loop 2 sequence to form a cyclic structure (ring); and "linker1" and "linker2" represent any chemically and biologically compatible covalent grouping of atom serving to link two monomeric loop 2 sequences and their associated constraints to give a dimeric, tricyclic peptide. These linkers may be the same or different

Generally, the preferred linking groups have 0 to 20 carbon atoms, and 0 to 10 heteroatoms (N, O, S, P etc.), and may be straight or branched, may contain saturated,

monomeric
loop 4 sequence
└constraint'┘

where:

monomeric loop 4 sequence represents a sequence of amino acid residues or functional equivalents thereof, which is sustantially homologous to the p75 binding region of loop 4 of BDNF, and comprises all or part of the following sequence:

Lys^{95}-Lys^{96}-Arg^{97}; and,

"constraint'" represents a conformational constraint imposed on the monomeric loop 4 sequence, which acts to limit the flexibility of the "monomeric loop 4 sequence", for example by covalently linking all or part of the "monomeric loop 4 sequence" to form a cyclic structure (ring).

The "constraint'" can be derived by several methods, including but not limited to:

(i) cyclising the N-terminal amine with the C-terminal carboxyl acid function, either directly via an amide bond between the N-terminal nitrogen and C-terminal carbonyl, or indirectly via a spacer group, such as one or more additional amino acid resdues, including α- and ω-amino carboxylic acid residues;

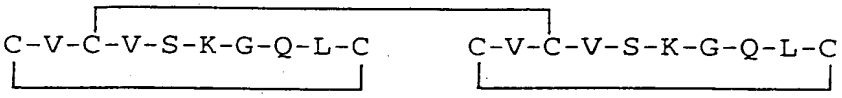
(ii) cyclising via the formation of a covalent bond between the side chains of two residues, such as an amide bond between between a lysine residue and either an aspartic acid or glutamic acid residue, or a disulphide bond between two cysteine residues, or a thioether bond between a cysteine residue and an ω-halogenated amino acid residue, either directly or via a spacer group as described in (i) above. The residues contributing the side chains may be derived from the "monomeric loop 4 sequence" itself, or may be incorporated into or added on to the "monomeric loop 4 sequence" for this purpose; and

design and synthesis of compounds of improved activity, stability and bioavailability.

Preferably where amino acid substitution is used, the substitution is conservative, *i.e.* an amino acid is replaced by one of similar size and with similar charge properties.

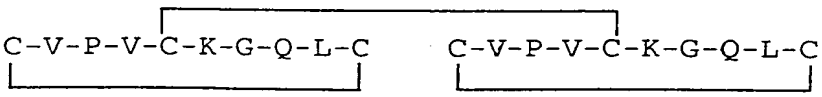
In particularly preferred embodiments the bicyclic dimer is:

$(L2\text{-}8P2C)_2$



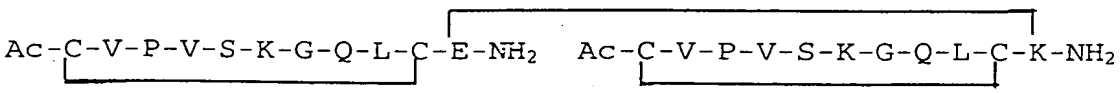
or

$(L2\text{-}8S4C)_2$



where the dimeric bicyclic peptides $(L2\text{-}8P2C)_2$ and $(L2\text{-}8S4C)_2$ consist of monomeric loop 2 sequences constrained by disulphide bonds formed between cysteine residues added to the loop 2 sequence and joined by a linker consisting of a disulphide bond formed between cysteine residues substituted into the loop 2 sequence, or

$(L2\text{-}8\&E+K)_2$



where the dimeric bicyclic peptide $(L2\text{-}8\&E+K)_2$ consists of monomeric loop 2 sequences constrained by disulphide bonds formed between cysteine residues added to the loop 2 sequence and joined by a linker consisting of an amide bond formed between a glutamate and a lysine residue added to the loop 2 sequence;

compositions are well known in the art, as set out in textbooks such as Remington's Pharmaceutical Sciences, 17th Edition, Mack Publishing Company, Easton, Pennsylvania, USA. Pharmaceutically-acceptable carriers include conventional carriers which are suitable for use with peptide-based drugs, including diluents, excipients, and preservatives and the like. For example, carriers such as dextrose, mannitol, sucrose, or lactose, buffer systems such as acetate, citrate and phosphate, and bulking agents such as serum albumin, preferably human serum albumin, may be used.

The invention also provides a culture medium additive for promotion of growth of neuronal cells *in vitro*, comprising a compound according to the invention together with a carrier or diluent which does not adversely effect the growth of cells in culture. Suitable carriers and diluents will be well known to the person skilled in art, and include physiologically acceptable fluids such as water, saline solution, or buffer solutions.

The optimal concentration of compound will vary according to the cell type and the culture conditions, but will generally be in the range 1-500μM, preferably 1-100μM.

The invention further provides a method of treatment of a condition characterised by neuronal deficit or neuronal death, comprising the step of administering an effective amount of a compound of the invention to a subject in need of such treatment.

It is contemplated that the method of the invention is suitable for treatment of conditions including but not limited to neurodegenerative diseases such as motor neurone disease (amyotrophic lateral sclerosis), progressive spinal muscular atrophy, infantile muscular atrophy, Charcot-Marie-Tooth disease, Parkinson's Disease, Parkinson-Plus syndrome, Guamanian Parkinsonian dementia complex, progressive bulbar atrophy, Alzheimer's disease and the like, other neurodegenerative conditions such as those arising from ischaemia, hypoxia, neural injury,

peptide synthesis method, and PIN synthesis methods (for review, see Maeji *et al.*, 1995). Those skilled in the art will readily be able to select the most suitable method for any given compound of the invention.

Brief Description of the Figures

Figure 1 shows the backbone trace of the three-dimensional structure of BDNF dimer (one monomer in black, the other grey), showing the positions of the loop 2 (trkB binding) and loop 4 (predominantly p75 binding) regions. Sidechains of the p75 binding tripeptide in loop 4 (Lys-Lys-Arg) are shown.

Figure 2 illustrates the molecular modelling of monomeric cyclic loop 2 analogues. An α-carbon to α-carbon trace of the native loop 2 of BDNF is shown, superimposed with low-energy conformations of loop 2 analogues L2-12, L2-10, L2-8 and L2-6, each of which is constrained by a disulphide bridge (indicated by arrows).

Figure 3 shows the concentration-response curves of monomeric cyclic loop 2 analogues in competition with BDNF. The monomeric cyclic loop 2 analogues L2-12 (closed triangles), L2-12a (open triangles), L2-10 (open squares), L2-8 (closed diamonds) and L2-6 (open diamonds) and the monomeric linear peptide L2-12b (closed squares) were assayed in competition with BDNF (4×10^{-11} M) in cultures of E8 to E10 chick sensory neurons. Surviving neurons were counted after 48 hrs in culture, and these counts were then expressed as a percentage of originally-plated viable neurons and normalised such that survival in cultures containing BDNF alone (P; positive control; closed circle) was set to 100% and survival in cultures with neither BDNF nor loop 2 analogue (N; negative control; open circle) to 0%. Data are expressed as the mean ± SEM from at least 8 observations (n=8) from 4 independent experiments.

Figure 4 shows the concentration-response curves of monomeric cyclic peptide L2-12, alone and in competition with NGF. Surviving neurons were counted after 48 hrs in

percentage of originally-plated viable neurons then normalised such that survival of cultures containing BDNF was set as 100%, while that for cultures with neither BDNF nor loop 2 analogue was set to 0%. Maximal inhibition of BDNF-mediated survival was calculated by subtracting the lowest value for BDNF-mediated survival from that of BDNF alone (100%). Note that alanine substitution in the L2-12 sequence can affect the ability of these peptides to modulate BDNF-mediated survival. Significant reduction in inhibitory activity, compared to L2-12 (closed bar), was observed when Ala was substituted for Val^3, Val^5, Ser^6 (***$p < 0.001$; ANOVA Bonferroni multiple comparisons test n=12), Lys^{11} (*$p < 0.05$; n=12) and Gln^{12} (**$p < 0.01$; n=10). No data (ND) were obtained for L2-12P4ΔA. Data are expressed as the mean ± SEM.

Figure 7A shows a schematic view of the two loop two regions in the model of the three-dimensional structure of the BDNF dimer, showing the interatomic distances (Å) between α-carbon atoms of selected residues.

Figure 7B shows a schematic view of the disulphide bridge of the cystine residue, showing the average interatomic distance and 90% confidence interval (90% CI) of α-carbon atoms, determined by conformational analysis.

Figure 8 shows a graph of the survival of sensory neurons in the presence of the bicyclic dimeric peptides $(L2\text{-}8P2C)_2$, $(L2\text{-}8V3C)_2$ and $(L2\text{-}8S4C)_2$. Neurons were prepared from chick dorsal root ganglia from embryonic chicks (E8-E10), and surviving neurons counted after 48 hours in culture. Data are presented as a percentage of the number of cells supported by BDNF (1ng/ml; 100%) after the same period in culture. Survival in negative control cultures was set to 0%. Highly significant differences in neuronal survival in the presence of $(L2\text{-}8P2C)_2$, and $(L2\text{-}8S4C)_2$ were observed compared to survival in negative controls (ANOVA, *** $p<0.001$, Bonferroni multiple comparisons test).

Neurons were prepared from dorsal root ganglia from embryonic chicks (E8-E10) and surviving neurons counted after 48 hours in culture. B: positive control (BDNF 1 ng/ml); N: negative control (no peptide). *** Significantly different to negative control (ANOVA, $p < 0.001$, Bonferroni mutiple comparisons test, $n = 12$).

Figure 13 shows a graph of the survival of sensory neurons in the presence of the monomeric cyclic loop 4-derived peptides L4-3pA(I), L4-3pA(II) and L4-3Hx, and their linear homologues L4-3pAa and L4-3Hxa. All peptides were added at a concentration of 10^{-6} M. BDNF was added at 1 ng/ml. Neg: shows the survival in negative control cultures containing neither BDNF nor peptide. *** Significantly different to negative control (ANOVA, $p < 0.001$, Bonferroni mutiple comparisons test, $n = 12$).

Figure 14 shows a graph of the effect of the monomeric cyclic loop 4-derived peptides L4-3pA(I) (open diamonds), L4-3pA(II) (open squares) and L4-3Hx (open triangles), and their linear homologues L4-3pAa (crosses) and L4-3Hxa (asterisks) on the neuronal survival effect mediated by BDNF (1 ng/ml). Over the concentration range tested (10^{-11} to 10^{-5}), none of the peptides exhibited a significant effect on BDNF mediated neuronal survival.

Figure 15 shows shows a graph of the effect of the monomeric cyclic loop 4-derived peptides L4-3pA(I) (open diamonds), L4-3pA(II) (open squares) and L4-3Hx (open triangles), and their linear homologues L4-3pAa (crosses) and L4-3Hxa (asterisks) on the neuronal survival effect mediated by NGF (1 ng/ml). Over the concentration range tested (10^{-11} to 10^{-5}), none of the peptides exhibited a significant effect on NGF mediated neuronal survival.

Figure 16 shows a graph of the survival of sensory neurons in the presence of the monomeric cyclic loop 4 peptides, L4-3Ap(I), L4-3Ap(II), L4-3AP(I) and L4-3AP(II). All peptides were added at a concentration of 10^{-6} M. BDNF was added at a concentration of 1ng/ml. Neg shows the survival of control cultures containing neither

Materials

Mouse recombinant BDNF was a kind gift from Dr R Kolbeck and Professor Y-A Barde (Max-Planck-Institute for Psychiatry, Martinsried, Federal Republic of Germany). NGF, purified from male mouse submaxillary gland was purchased from Boehringer-Mannheim (Mannheim, Federal Republic of Germany). Fertilised chicken eggs were obtained from Research Poultry Farms (Research, Victoria, Australia), trypsin from Worthington (Freehold, NJ, U.S.A.), L-15 from GIBCO BRL (Grand Island, NY, U.S.A.), horse serum from CSL (Parkville, Victoria, Australia), Nunclon 10 cm tissue culture dishes from Nalge Nunc International (Roskilde, Denmark), Falcon Multiwell 48-well tissue culture plates from Becton Dickinson (Franklin Lakes, NJ, U.S.A.) and mouse laminin, isolated from Englebreth-Holm-Swarm tumour cells, from Collaborative Biomedical Products (Bedford, MA, U.S.A.). Fmoc-amino acids and Wang resin were purchased from Auspep (Parkville, Victoria, Australia), PR-500 resin from Calbiochem-NovaBiochem (Alexandria, New South Wales, Australia) and Econosil irregular packed HPLC columns from Alltech Associates (Baulkham Hills, New South Wales, Australia). Other reagents were purchased from Sigma (Castle Hill, New South Wales, Australia).

Example 1 Homology Modelling of BDNF

A model of the three-dimensional structure of murine BDNF was obtained by protein homology modelling techniques from murine NGF. This was performed by the Swiss-Model automated protein homology server running at the Glaxo Institute for Molecular Biology in Geneva, Switzerland, accessed via the Internet (http://expasy.hcuge.ch/swissmod/SWISS-MODEL.html, Peitsch, 1995). Briefly, a three-dimensional model of the target sequence is produced in the following manner: Swiss-Model searches the Brookhaven Protein Data Bank for the sequences of homologous proteins of known three-dimensional

geometrically optimised using the Polak-Ribiere algorithm and MM+ forcefield to a local low-energy conformation. These modelled peptides were assessed for their ability to mimic the native conformation by measuring the root mean square deviation of the peptide backbone to that of the native loop following least squares superimposition.

From this model of BDNF the second β-hairpin loop (loop 2) was defined as Glu^{40}-Lys^{41}-Val^{42}-Pro^{43}-Val^{44}-Ser^{45}-Lys^{46}-Gly^{47}-Gln^{48}-Leu^{49}-Lys^{50}-Gln^{51}, where the amino acid numbering, is the same as in mature BDNF. Peptide analogues of this loop were modelled to investigate

(i) what type of constraint would be most appropriate to allow the peptides to mimic loop 2 in its native loop conformation, and

(ii) where in the sequence this constraint would be best positioned.

As a result of these studies four peptides, L2-12, L2-10, L2-8 and L2-6, each constrained by a disulphide bridge between terminal cysteine residues, were chosen for synthesis and biological examination. The sites from which these peptides are derived are illustrated in Figure 2.

Example 3 Synthesis of Monomeric Cyclic Loop 2 Peptides

Linear peptides in the free acid form were assembled manually from Fmoc-amino acids on Wang resin using batch-type solid phase methods (Fields and Noble, 1990). Both coupling and deprotection reactions were assessed with the trinitrobenzenesulphonic acid test (Thompson *et al*, 1995). The linear peptide amide L2-12a was synthesised using continuous flow methods on PR-500 resin; coupling and deprotection steps were monitored spectrophotometrically. Cleavage of peptides from the resin and sidechain removal was accomplished with trifluoroacetic acid (TFA)/ethanedithiol/H_2O (18:1:1).

Crude peptide products were analysed and purified by reversed phase HPLC over Econosil C-18 irregular packed

Table 1

Monomeric Cyclic Loop 2 Analogue Sequences

Code	Peptide analogue sequence
L2-12	C-E-K-V-P-V-S-K-G-Q-L-K-Q-C
L2-12a	Ac-C-E-K-V-P-V-S-K-G-Q-L-K-Q-C-NH_2
L2-12b	E-K-V-P-V-S-K-G-Q-L-K-Q
L2-10	C-K-V-P-V-S-K-G-Q-L-K-C
L2-8	C-V-P-V-S-K-G-Q-L-C
L2-6	C-P-V-S-K-G-Q-C
L2-12E1ΔA	C-**A**-K-V-P-V-S-K-G-Q-L-K-Q-C
L2-12K2ΔA	C-E-**A**-V-P-V-S-K-G-Q-L-K-Q-C
L2-12V3ΔA	C-E-K-**A**-P-V-S-K-G-Q-L-K-Q-C
L2-12P4ΔA[a]	C-E-K-V-**A**-V-S-K-G-Q-L-K-Q-C
L2-12V5ΔA	C-E-K-V-P-**A**-S-K-G-Q-L-K-Q-C
L2-12S6ΔA	C-E-K-V-P-V-**A**-K-G-Q-L-K-Q-C
L2-12K7ΔA	C-E-K-V-P-V-S-**A**-G-Q-L-K-Q-C
L2-12G8ΔA	C-E-K-V-P-V-S-K-**A**-Q-L-K-Q-C
L2-12Q9ΔA	C-E-K-V-P-V-S-K-G-**A**-L-K-Q-C
L2-12L10ΔA	C-E-K-V-P-V-S-K-G-Q-**A**-K-Q-C
L2-12K11ΔA	C-E-K-V-P-V-S-K-G-Q-L-**A**-Q-C
L2-12Q12ΔA	C-E-K-V-P-V-S-K-G-Q-L-K-**A**-C

Amino acids are represented by their one letter code, reading left to right from amino to carboxyl termini. The analogue code, for example L2-12K9ΔA, refers to 12 residues from the native loop 2 sequence of BDNF with lysine at position 9 substituted with alanine. Cysteine residues not found in the native BDNF sequence were incorporated to form disulphide bridges, which are represented by lines between side chains.

[a]peptide L2-12P4ΔA not synthesized.

experiments were analysed for lack of significant variation using a parametric one-way analysis of variance (ANOVA) before being grouped. Statistics were performed using Instat version 2.04a (GraphPad, San Diego, CA, U.S.A.). Prism software (GraphPad, San Diego, CA, U.S.A.) was used to fit sigmoidal curves to the data.

To investigate the ability of the loop 2 analogues L2-12, L2-12a, L2-10, L2-8 and L2-6 to modulate BDNF-mediated survival, the peptides were assayed from 1×10^{-11} to 1×10^{-4} M in competition with BDNF at 4×10^{-11} M, a concentration which produces near maximal survival. The results are summarised in Figure 3. All five peptides showed a similar pattern of concentration-dependent inhibition of BDNF-mediated survival, causing an increase in inhibition from 1×10^{-11} to a maximum at approximately 1×10^{-6} M; above this concentration inhibition either reached a plateau (L2-10), or diminished (L2-12, L2-12a, L2-8 and L2-6), giving the concentration-response curve an inverted bell-shape. However, the maximal level of inhibition produced by these peptides varied: L2-8 showed the greatest maximum (50% ± 5), followed by L2-12a (44% ± 4), L2-10 (41% ± 2), L2-12 (40% ± 3) and L2-6 (27% ± 6). The maximal inhibition and pIC_{50} values, the latter obtained from logistic sigmoidal curves fit to the data, are summarised in Table 2.

In contrast to the results obtained with the monomeric cyclic peptides, the monomeric linear peptide L2-12b did not show significant inhibition of BDNF-mediated survival over the concentration range tested (1×10^{-11} to 1×10^{-4} M; Figure 3).

Example 5 Specificity of Inhibition of Neuronal Survival Activity by Monomeric Cyclic Loop 2 Analogues

To determine the specificity of the peptides in inhibiting BDNF-mediated survival, monomeric cyclic peptide L2-12 (1×10^{-11} to 1×10^{-4} M) was assayed in competition with NGF (4×10^{-11} M), using the assay described in Example 4. As shown in Figure 4, at the concentrations tested peptide L2-12 did not significantly inhibit NGF-mediated survival. These data suggest that the inhibition of neuronal survival seen in Example 4 is specific for BDNF.

Example 6 Lack of Intrinsic Neuronal Survival Activity or Toxic Effects of Monomeric Cyclic Loop 2 Analogues

When added to cultures alone, *ie.* in the absence of neurotrophin, the monomeric cyclic peptide L2-12 neither intrinsically promoted neuronal survival nor exhibited non-specific toxic effects on neurons at the concentrations tested (1×10^{-11} to 1×10^{-4} M), giving neuronal survival of around 5%, *ie.* similar to that of negative controls, as shown in Figure 4.

This lack of intrinsic neuronal survival promoting activity of the monomeric cyclic loop 2 peptide L2-12 was expected. Because L2-12 and the other monomeric cyclic loop 2 peptides are monomeric, they are unlikely to dimerise trkB, and the dimerization is crucial for trk-mediated signalling (Jing *et al*, 1992).

BDNF (data not shown). No significant change in maximal inhibition was observed when Ala was substituted for $Glu^{1(40)}$ (39% ± 13), $Lys^{2(41)}$ (26% ± 7), $Lys^{7(46)}$ (33% ± 8), $Gly^{3(47)}$ (46% ± 13), $Gln^{9(48)}$ (32% ± 6) and $Leu^{10(49)}$ (33% ± 6).

Example 9 Molecular Design of Disulphide-linked Dimeric Bicyclic Loop 2 Analogues

The two loop 2 regions of BDNF are juxtaposed in the three-dimensional model of the dimer (Figure 1), which allows design of small dimeric peptides which mimic this spatial arrangement. On the basis of observations made in the highly analogous NGF-trkA receptor system, we predicted that small dimeric loop 2 analogues could act as agonists if they could facilitate dimerisation of trkB. It has been shown that divalent antibodies to trkA can cause the homodimerisation of this receptor, leading to signal transduction and NGF-like biological activity in vitro (Clary *et al*, 1994). Moreover, a small peptide mimetic of erythropoietin, produced by a recombinant library technique, possesses full erythropoietin-like biological activity as a result of self-association to form a dimer which dimerises the erythropoietin receptor (Wrighton *et al*, 1996). Examination of the X-ray crystal structure of the peptide-erythropoietin receptor complex (Livnah *et al*, 1996) reveals that the structure of the bound dimeric peptide bears a striking resemblance to the loop 2 regions of BDNF in our three-dimensional model.

The most effective of the monomeric, cyclic, disulphide-linked loop 2 peptides which were shown in Examples 4, 5, 7 and 8 were able to inhibit BDNF neuronal survival activity, peptide L2-8, was chosen as the basis for the design of dimeric peptides. This peptide consists of 8 amino acid residues of BDNF plus the two terminal cysteine residues oxidised to cyclic disulphide, *ie*. a total of 10 residues.

Example 10 Synthesis of Disulphide-Linked Dimeric Bicyclic Loop 2 Analogues

The disulphide-linked dimeric bicyclic peptides $(L2\text{-}8P2C)_2$, $(L2\text{-}8V3C)_2$ and $(L2\text{-}8S4C)_2$ were synthesised by standard solid phase synthesis techniques using Fmoc amino acids, as described in Example 3, and using a mixed Cys protection strategy (Cys(Trt) and Cys(Acm)). The general method is illustrated in Scheme 1.

Peptides and intermediates were purified by reverse-phase high performance liquid chromatography, and characterised by electrospray mass spectrometry. The structures of the compounds synthesised are shown in Table 3.

Example 11 Intrinsic Neuronal Survival Activity of Disulphide-Linked Dimeric Bicyclic Loop 2 Analogues

The disulphide-linked dimeric bicyclic loop 2 peptides were analysed for their ability to promote the survival of sensory neurons in cultures prepared from dorsal root ganglia obtained from embryonic day 8-10 chicks, as described in Example 4. Peptides $(L2\text{-}8P2C)_2$ and $(L2\text{-}8S4C)_2$ each displayed concentration-dependent neuronal survival activity, maximally promoting the survival of 28% and 30% of the neurons that would be supported by BDNF itself. These results are shown in Figure 8.

The activity of these two disulphide-linked dimeric bicyclic loop 2 peptides was surprising in view of the number of the modifications. In peptide $(L2\text{-}8S4C)_2$, the serine residue (Ser^4) shown to be important for the inhibitory action on BDNF-mediated neuronal survival of the monomeric cyclic loop 2 peptides (see Example 8) was replaced by the disulphide-linked cystine residue. In peptide $(L2\text{-}8P2C)_2$, the Cα-to-Cα distance of the cystine residue is likely to be much shorter than the corresponding distance in our model of BDNF of the two proline residues which it replaces.

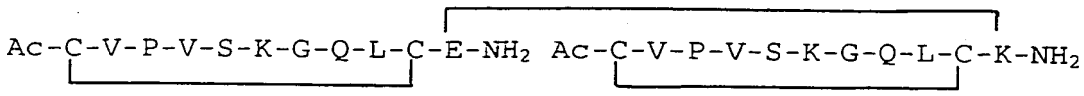
In contrast, peptide $(L2\text{-}8V3C)_2$ was inactive, as shown in Figure 8, despite the likelihood that it could best accommodate the cystine residue, at least in terms of interatomic distance.

Example 12 Lack of Intrinsic Neuronal Survival Activity of Monomeric Precursors of Dimeric Bicyclic Loop 2 Analogues

To determine whether the dimeric nature of the disulphide-linked dimeric bicyclic loop 2 peptides was required for intrinsic neuronal survival activity, we assayed peptides L2-8P2C(Acm) and L2-8S4C(Acm), the monomeric cyclic precursors of peptides $(L2\text{-}8P2C)_2$ and $(L2\text{-}8S4C)_2$ in which the Acm groups on the internal Cys were

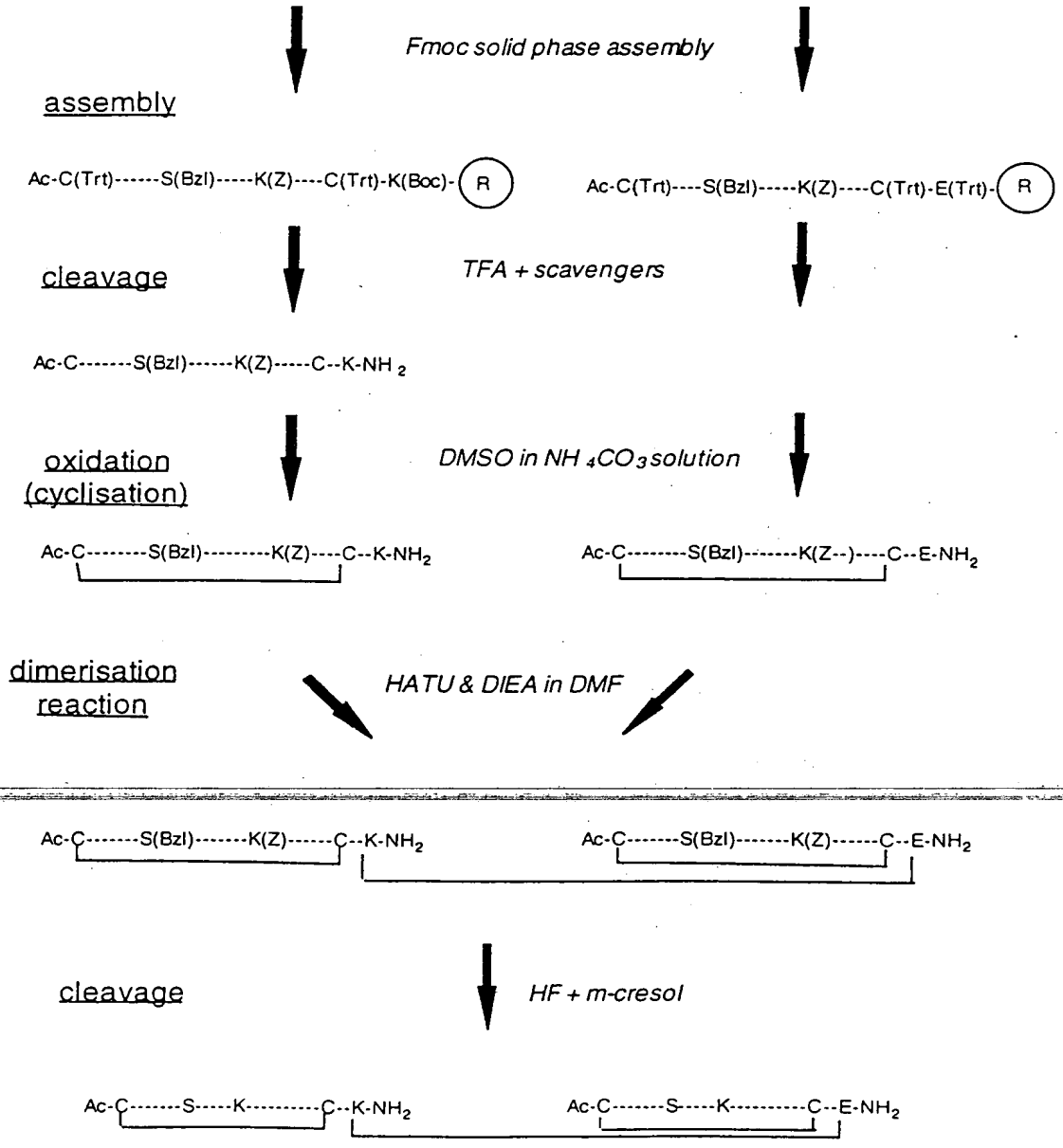
Table 4 Structure of disulphide-linked bicyclic loop 2 analogue

$(L2\text{-}8\&E+K)_2$



Scheme 2

Synthesis of an amide-linked dimeric bicyclic loop 2 analogue



create a molecule that might show either improved efficacy (as evidenced by an increase in the maximal percent neuronal survival) or increased potency, we reasoned that we would need to restrict the freedom of rotation about the dimerising constraint. To do this, we chose to combine, in one molecule, the two different dimerising constraints used in the disulphide-linked and the amide-linked dimeric bicyclic loop 2 analogues. We anticipated that the resultant dimeric tricyclic loop 2 analogue $(L2\text{-}8S4C\&E+K)_2$, by restricting the rotation of the two loop 2 moieties relative to one another would much better mimic the loop 2 orientation seen in the native protein, and therefore would show improved efficacy and potency. This is shown in Table 5.

Example 17 Synthesis of a Dimeric Tricyclic Loop 2 Analogue

The dimeric tricyclic peptide $(L2\text{-}8S4C\&E+K)_2$ was prepared as shown in Scheme 3 from two cyclic N-acetylated, C-amidated, partially-protected monomers synthesised by standard solid phase techniques on Rink amide MBHA resin as described in Example 14.

The monomers were initially condensed in the first dimerisation reaction via the free lysine and glutamate sidechains. A second dimerisation reaction was carried by oxidising the internal cysteine residues, completing the tricycle. The remaining Lys protecting groups were removed by treating the partially protected tricyclic dimer with hydrogen fluoride/m-cresol (10:1) for one hour at 5°C. HF was removed by evaporation at room temperature. The desired peptide $(L2\text{-}8S4C\&E+K)_2$ and intermediates were purified by HPLC and characterised by mass spectrometry.

Example 18 Intrinsic Neuronal Survival Activity of a Dimeric Tricyclic Loop 2 Analogue

The dimeric tricyclic loop 2 peptide $(L2\text{-}8S4C\&E+K)_2$ was assayed in cultures of sensory neurons prepared from dorsal root ganglia obtained from embryonic chicks, as described in Example 4. Peptide displayed concentration dependent neuronal survival activity, supporting the survival of 35% of those neurons supported by BDNF (1ng/ml) with an EC_{50} in the order of 10^{-10} M. The results are shown in Figure 11.

The maximal neuronal survival promoting effect of the dimeric tricyclic loop 2 peptide $(L2\text{-}8S4C\&E+K)_2$ is similar to that of of the dimeric bicyclic loop 2 analogues. However peptide $(L2\text{-}8S4C\&E+K)_2$ is approximately two orders of magnitude more potent than the dimeric bicyclic analogues. This activity is consistent with the hypothesis that the presence of two dimerising constraints (Cys-to-Cys disulphide and Lys-to-Glu amide) would create a molecule which much better mimics the spatial arrangement of the two loop 2 moieties than any of the dimeric bicyclic compounds, which contain only a single dimerising constraint.

(HOBt) and diisopropylamine (DIEA) (HBTU:HOBt:DIEA 1:1:1.5 equivalents relative to peptide). Treatment of the product of this reaction with TFA/scavengers yielded the desired fully-deprotected product. The corresponding linear homologues were prepared by treating the appropriate sidechain-protected linear peptide with TFA/scavengers, without prior cyclisation.

Cyclisation reactions were monitored and peptides purified by reverse phase HPLC on either analytical (4.6 mm internal diameter) or semi-preparative (22.5 mm) C18 columns, using linear acetonitrile gradients in 0.1% TFA solution at appropriate flow rates. Desired fractions were collected and lyophilised for characterisation by mass spectrometry.

Synthesis of peptide L4-3pA yielded two stereoisomers, L4-3pA(I) and L4-3pA(II), each with the desired molecular weight of 581 daltons. These isomers were purified by HPLC and were assayed seperately for biological activity.

A list of the compounds synthesised is given in Table 6.

Example 21 Intrinsic Neuronal Survival Activity of a Monomeric Cyclic Analogue of the p75 Binding Region of Loop 4 of BDNF

The monomeric cyclic analogues of the p75 binding region of loop 4, L4-3pA(I), L4-3pA(II), L4-3Hx and their linear homologues L4-3pAa and L4-3Hxa, were assayed in cultures of sensory neurons prepared from embryonic chicks as described in Example 4. As shown in Figure 10, the monomeric cyclic loop 4 peptide, L4-3pA(II) displayed concentration-dependent neuronal survival activity. This intrinsic neuronal survival activity of L4-3pA(II) was surprising; unlike the loop 2 peptides decribed in Example 11, it is neither dimeric nor bicyclic. Moreover, the activity was confined to L4-3pA(II). Neither its stereoisomer L4-3pA(I), the other monomeric cyclic loop 4 peptide L4-3Hx constrained by the more conformationally flexible aminohexanoyl residue, nor their linear counterparts displayed neuronal survival activity in this assay system, as shown in Figure 11.

Example 22 Lack of Inhibition of BDNF-Mediated Sensory Neuron Survival by Monomeric Cyclic Loop 4 Analogues of the p75 Binding Region of Loop 4 of BDNF and Their Linear Homologues

The monomeric cyclic analogues of the p75 binding region of loop 4 L4-3pA(I), L4-3pA(II), L4-3Hx and their linear homologues L4-3pAa and L4-3Hxa, were assayed for their ability to modulate the neuronal survival effects of BDNF and NGF in cultures of sensory neurons prepared from embryonic chicks as described in Example 4. Unlike the monomeric cyclic loop 2 peptides, none of the monomeric cyclic loop 4 peptides or their linear homologues showed any significant inhibition of either BDNF- or NGF- mediated neuronal survival, as shown in Figures 12 and 13, respectively.

Table 7 Structure of Further Monomeric Cyclic Loop 4 Analogues

L4-3Ap(I) and L4-3Ap(II)	⌐Ala-DPro-Lys-Lys-Arg⌐
L4-3AP(I) and L4-3AP(II)	⌐Ala-Pro-Lys-Lys-Arg⌐

Table 8 Structure of Further Monomeric Cyclic Loop 4 Analogues Incorporating Ala for Lys

L4-3K3pA	[DPro-Ala-Ala-Lys-Arg]
L4-3K4ApA	[-DPro-Ala-Lys-Ala-Arg]

transformed using NMRpipe and analysed using NMRview. Complete assignment of all non-exchangeable proton resonances was made. Dihedral constraints for the backbone f angles were derived from the J^{3}_{NH-CaH} coupling constants measured from 1D spectra. A total of 61 structurally important distance constraints and 3 backbone f angle constraints were determined from the NMR data for L4-3pA(II).

Structure calculation was carried out using the software package DYANA. Cyclisation of the peptide was achieved by introducing a set of special distance constraints to both bring the ends of the peptide together and restrain the peptide bond angle to 180º. A modified version of the residue library containing a set of parameters defining a DPro residue was produced to allow calculation to include the DPro residue. A total of 100 structures were calculated on the basis of the NMR-derived constraint list by 10000 steps of simulated annealing followed by 2000 steps of minimisation of the DYANA target function. The 20 structures with the lowest target function were then selected as the final family of structures for the peptide. An overlay of these structures of peptide L4-3pA(II) can be found in Figure 18.

As can be seen in Figure 18, the conformation of the backbone of peptide peptide L4-3pA(II) is uniquely defined in solution. In addition, sidechain of Lys^{4} is adopts a single conformation up to its gamma-carbon atom, while the conformation of the sidechain of Arg^{5} is uniquely defined to the delta-nitrogen. The presence of a single backbone conformation and well-defined sidechains for peptide L4-3pA is consistent with the biological data showing that compounds of closely related sequence to L4-3pA show either markedly reduced or no neuronal survival activity in cell culture experiments. This exceptionally well-defined conformation of L4-3pA will be used as a template for the design of non-peptidic molecules with neuronal survival promoting activity.

specification.

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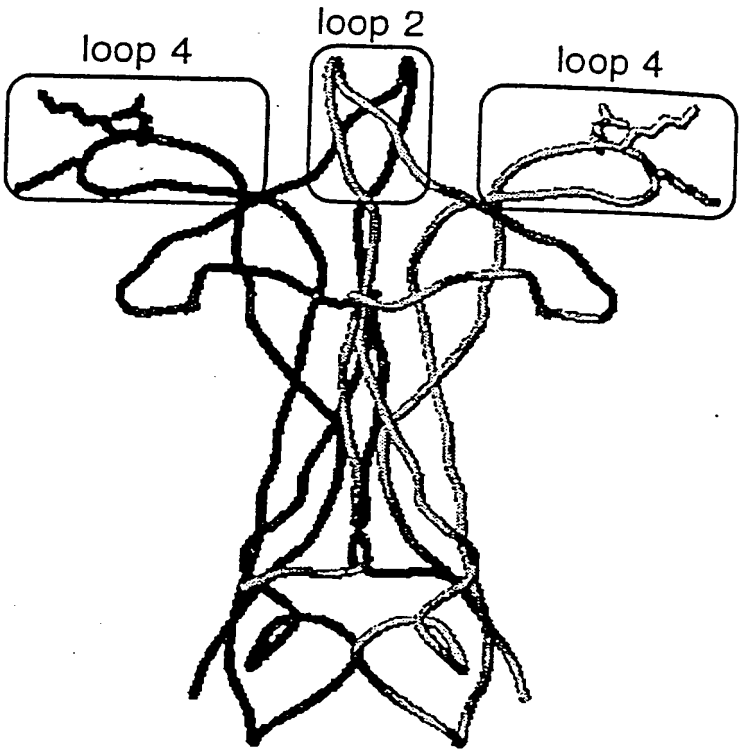

FIGURE 1

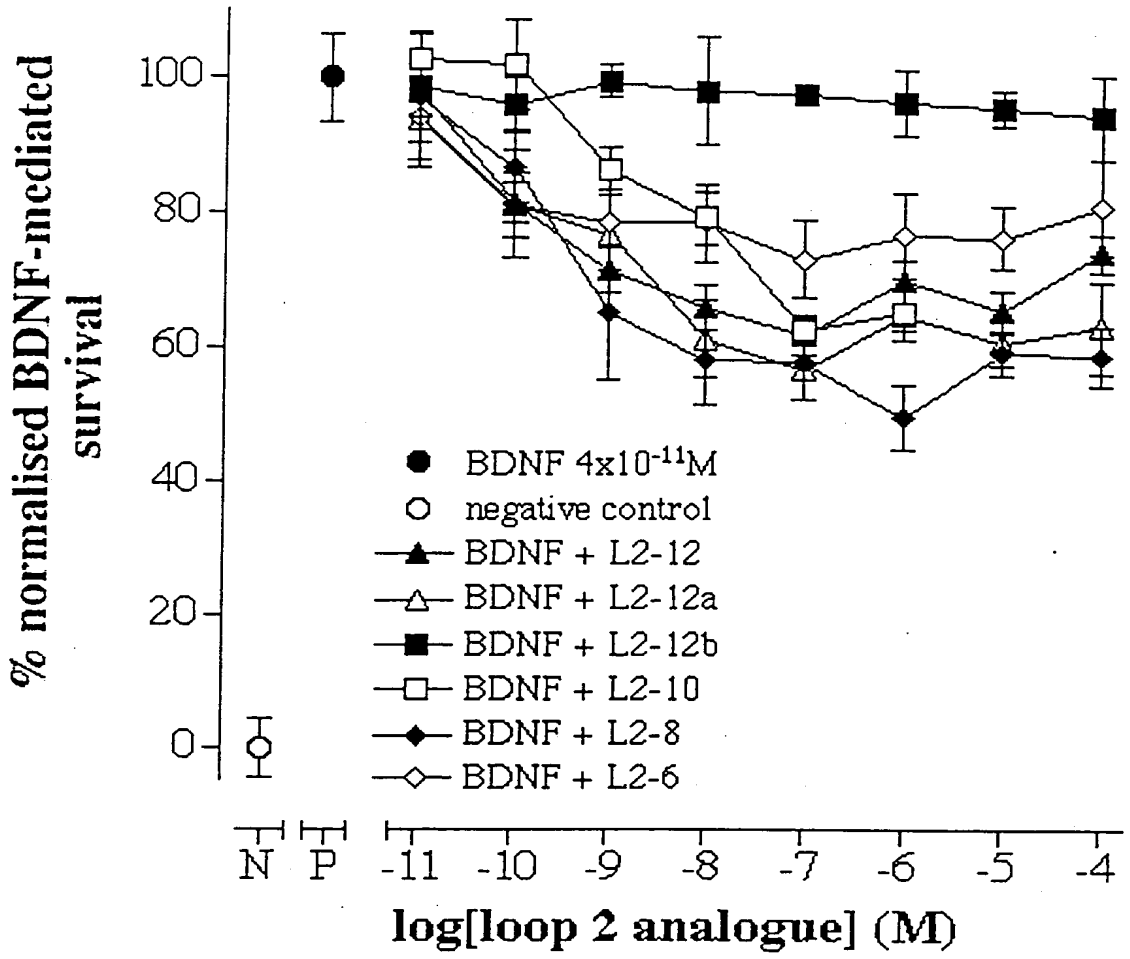
% normalised BDNF-mediated survival
100
80
60
40
20
0
BDNF 4x10-11M
negative control
BDNF + L2-12
BDNF + L2-12a
BDNF + L2-12b
BDNF + L2-10
BDNF + L2-8
BDNF + L2-6
N
P
-11
-10
-9
-8
-7
-6
-5
-4
log[loop 2 analogue] (M)

FIGURE 3

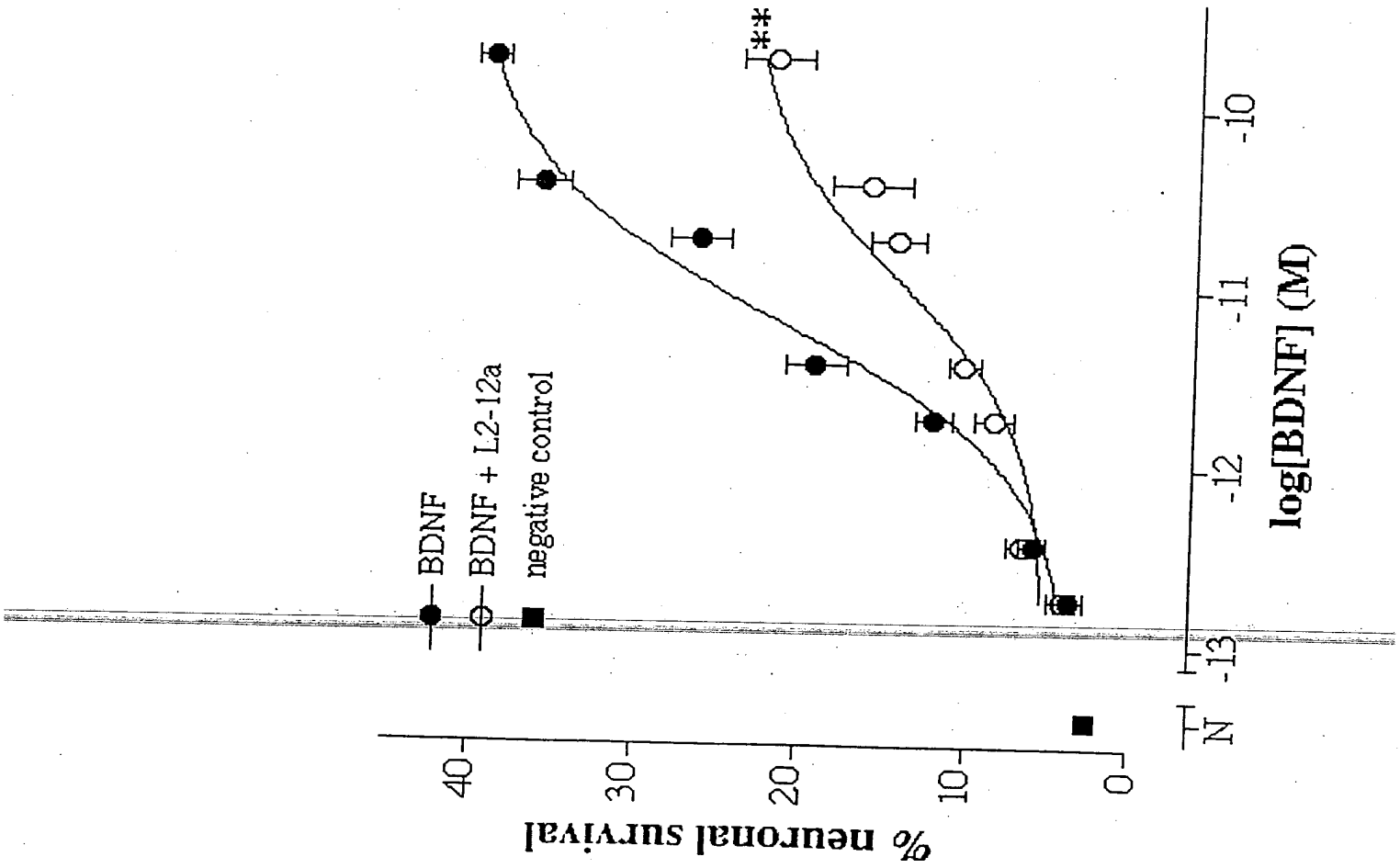
% neuronal survival
0
10
20
30
40
N
-13
-12
-11
-10
log[BDNF] (M)
**
BDNF
BDNF + L2-12a
negative control

FIGURE 5

Schematic view of loop 2 region of BDNF

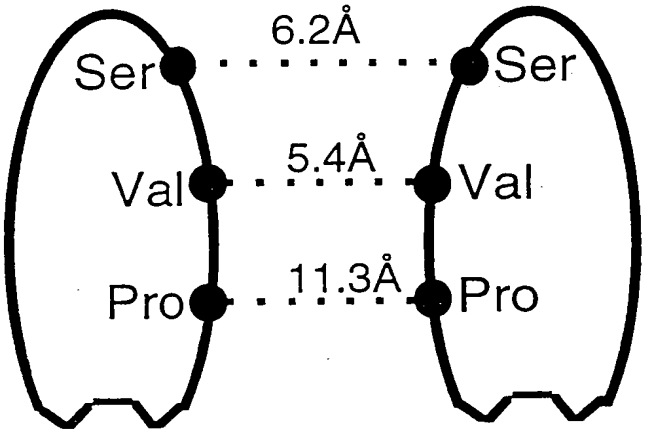

FIGURE 7A

Schematic view of disulphide bridge

average 5.3Å 90%CI 5.22-5.44Å

FIGURE 7B

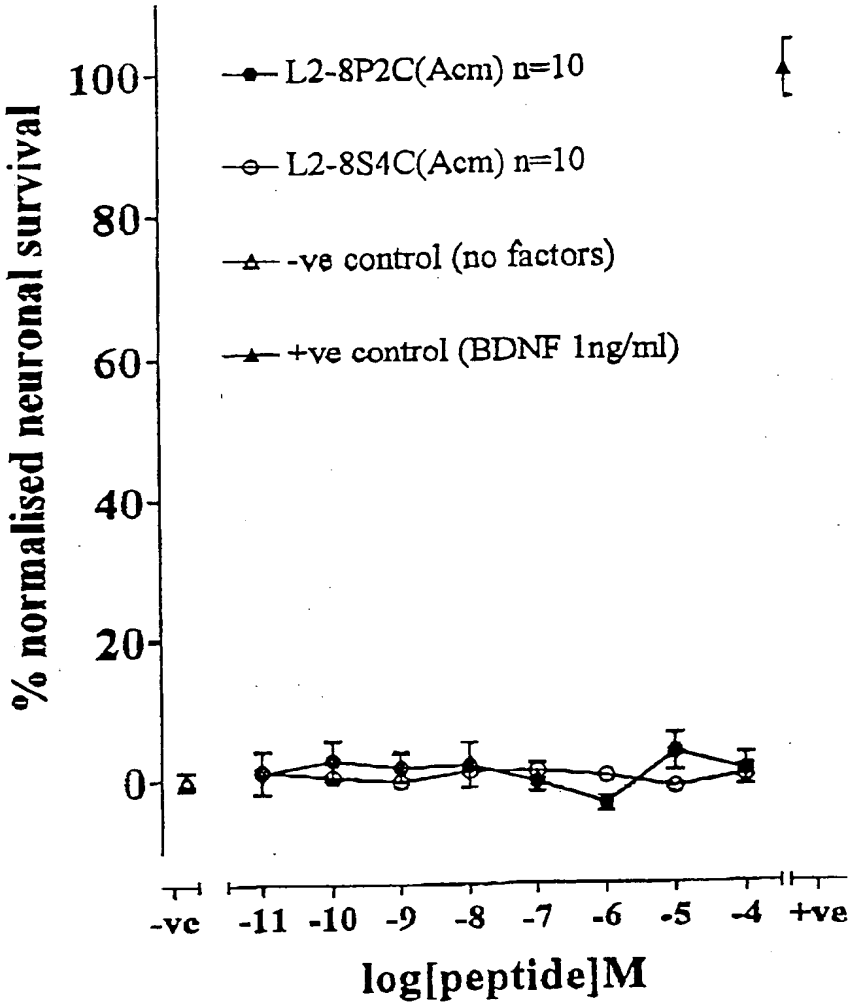
% normalised neuronal survival
100
80
60
40
20
0
L2-8P2C(Acm) n=10
L2-8S4C(Acm) n=10
-ve control (no factors)
+ve control (BDNF 1ng/ml)
-ve
-11
-10
-9
-8
-7
-6
-5
-4
+ve
log[peptide]M

FIGURE 9

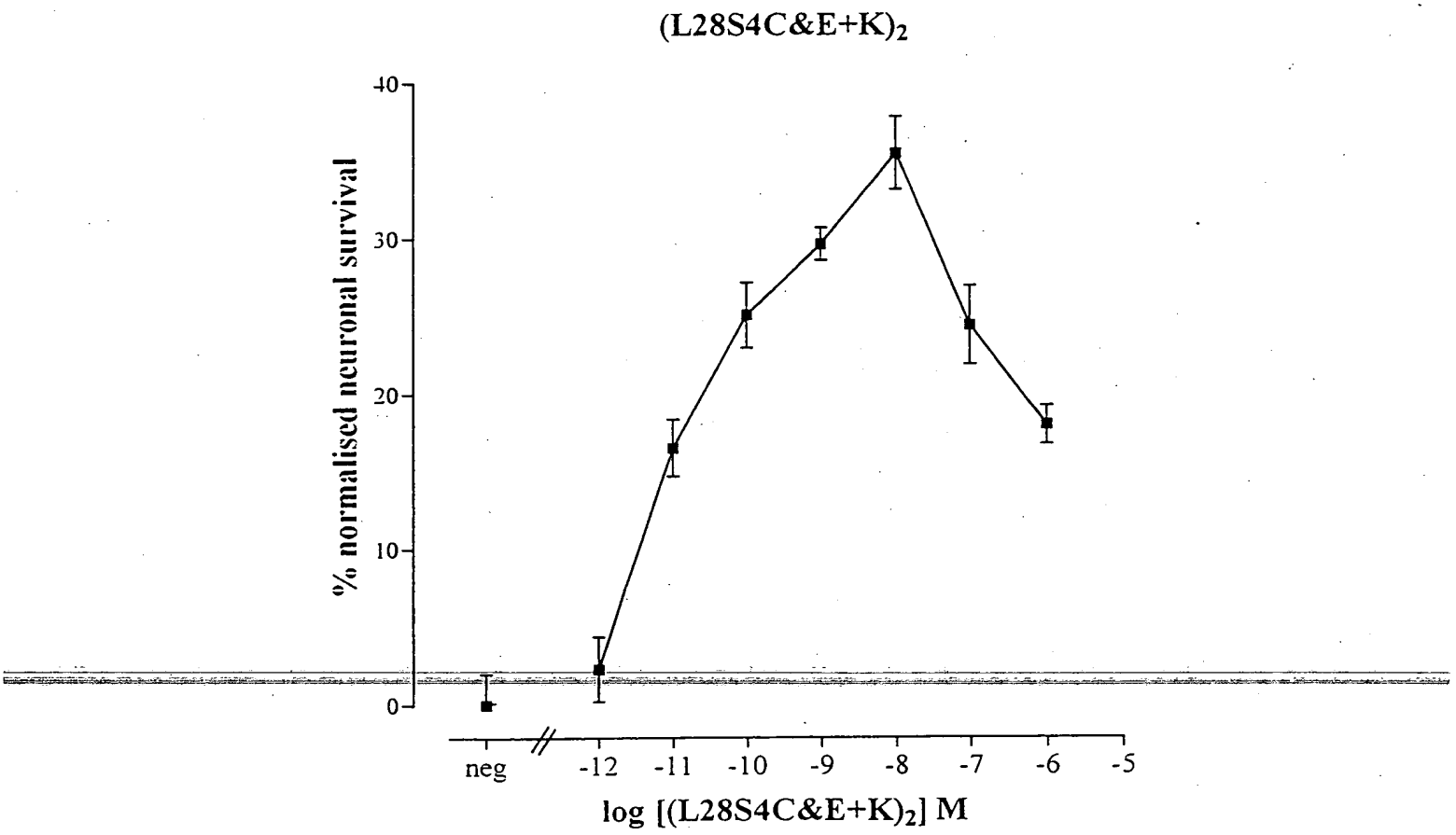
(L28S4C&E+K)2
% normalised neuronal survival
40
30
20
10
0
neg
-12
-11
-10
-9
-8
-7
-6
-5
log [(L28S4C&E+K)2] M

FIGURE 11

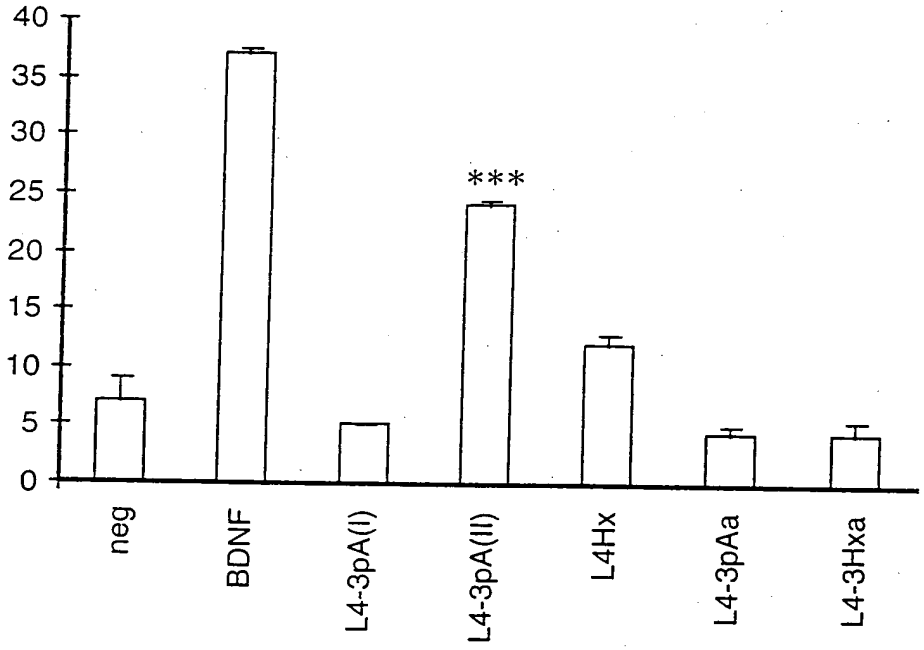
40
35
30
25
20
15
10
5
0
***
neg
BDNF
L4-3pA(I)
L4-3pA(II)
L4Hx
L4-3pAa
L4-3Hxa

FIGURE 13

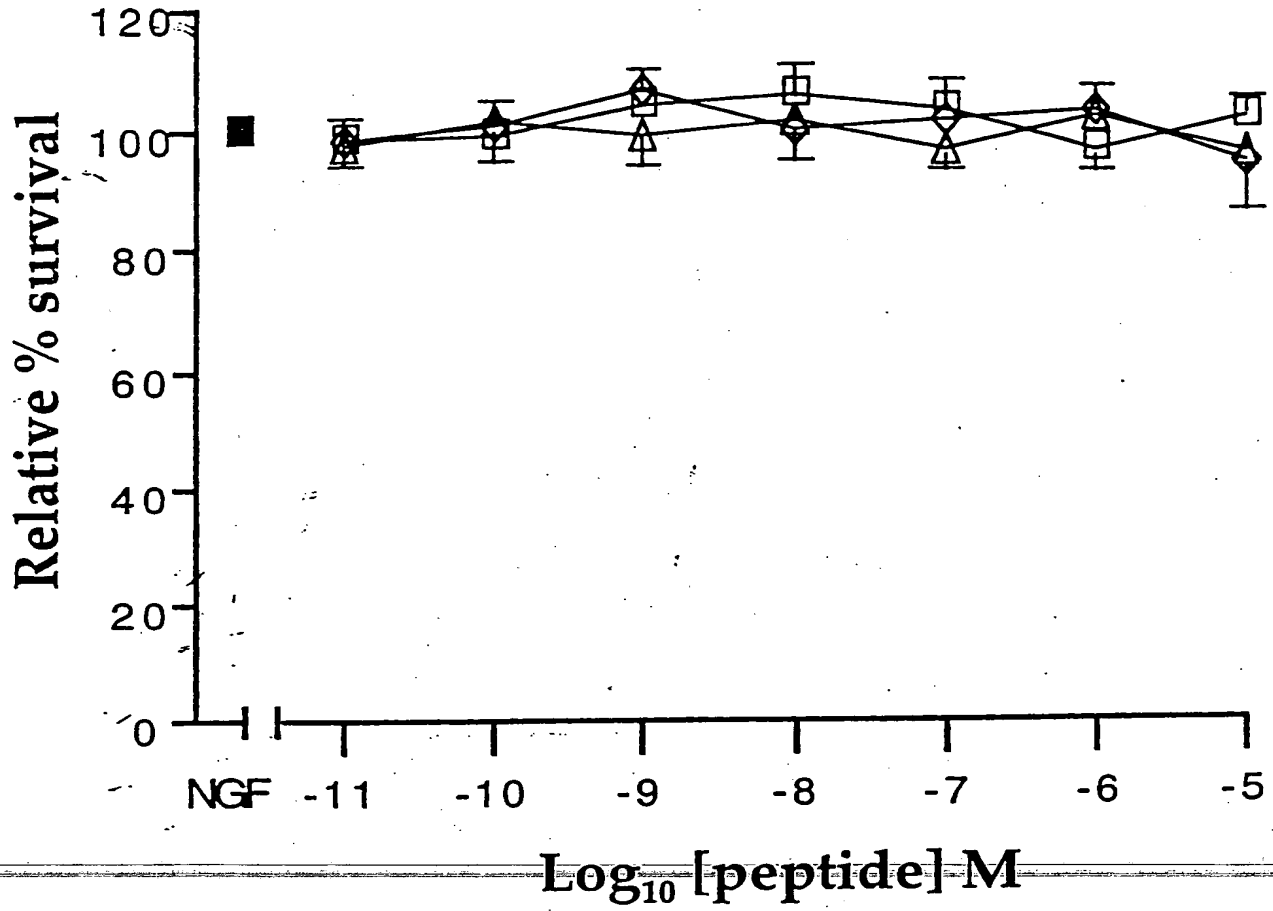
Relative % survival
120
100
80
60
40
20
0
NGF
-11
-10
-9
-8
-7
-6
-5
Log10 [peptide] M

FIGURE 15

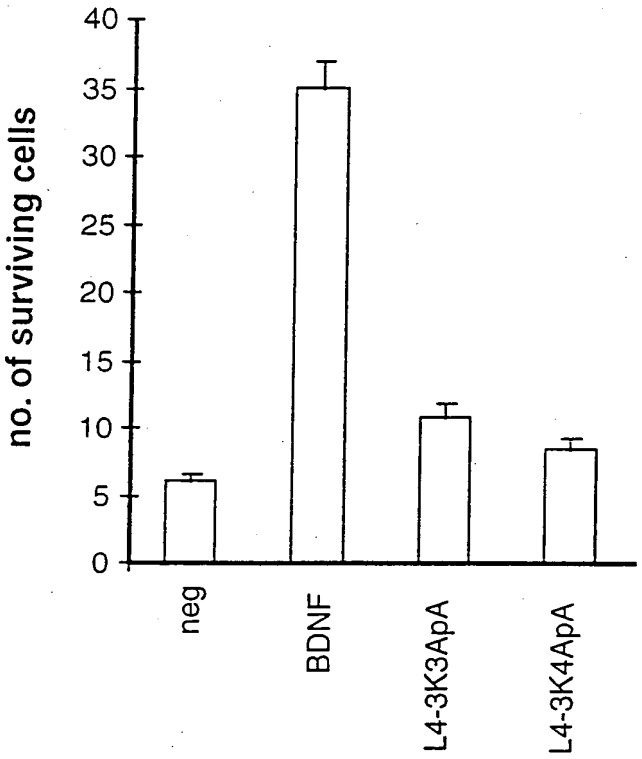
no. of surviving cells
40
35
30
25
20
15
10
5
0
neg
BDNF
L4-3K3ApA
L4-3K4ApA

FIGURE 17